Leukemic stem cells and measurable residual disease in AML

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CHAPTER 4

Absence of leukaemic CD34+ cells in acute myeloid leukaemia is of high prognostic value: a longstanding controversy deciphered

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Abstract

Primary resistance and relapses after initial successful treatment are common in acute myeloid leukaemia and therefore outcome remains poor. More accurate risk group stratification and effective personalized risk adapted treatment are necessary to improve outcome. The last two decades, controversial results have been published concerning the prognostic relevance of CD34 expression. In 706 acute myeloid leukaemia patients, we established a new flow cytometric-based CD34-definition, without use of cut-off values. We discriminate CD34-positive (n=548) and CD34-negative (n=158) patients by the presence or absence of neoplastic CD34+ cells, respectively. CD34-status was defined using aberrant immunophenotypes and validated using molecular phenotypes. This new definition of CD34 enables strong prediction of treatment outcome in the entire patient group and in several risk subgroups. Previously observed discrepancies in prognostic impact of CD34 protein expression using cut-offs (5%-20%) can now entirely be explained by considering the number of CD34-negative cases. In the total patient group the absence of neoplastic CD34-positive cells is paralleled by low levels of minimal residual disease, suggesting relative therapy sensitivity and explaining longer survival. Overall, we present CD34 surface expression as a relatively simple, powerful and independent predictor of clinical outcome, now warranting incorporation in acute myeloid leukaemia risk stratification.

Introduction

Acute myeloid leukaemia (AML) is a haematological malignancy, whereby undifferentiated cells of the myeloid lineage accumulate in the bone marrow (BM). Although up to 80% of the patients (<65yrs) achieve complete remission (CR) after intensive chemotherapy, around 40% will eventually relapse due to the outgrowth of minimal residual disease (MRD).[1]

Primary resistance and relapses after initial remission are recognized as the key problems in the treatment of AML. Risk group stratification helps to identify patients with a good, intermediate and poor prognosis. Applying risk-adapted therapy, whereby high risk patients receive a more intense treatment like an allogeneic stem cell transplantation, and good risk patients are spared from such toxic treatments, could therefore attribute to the improvement of survival rates. Current risk group stratification is based on different parameters available at diagnosis, including particular cytogenetic and molecular aberrancies, white blood cell (WBC) count and age. Response to treatment is one of the few post-diagnosis prognostic factors. Despite risk stratification, patients often perform different than predicted, for instance, there are good risk patients who relapse and poor risk patients who are cured. Since this can translate into under-treatment and over-treatment, respectively, there is a definite need for additional prognostic markers to refine current risk stratification, ultimately resulting in more personalized treatment, with consequent improvement of overall survival (OS). For many years there has been controversy concerning the prognostic impact of CD34 expression in AML. CD34 is a membrane glycoprotein that has been linked to increased resistance to apoptosis.[2–5] Multidrug transporters, like P-glycoprotein (Pgp), have been shown to be higher expressed at higher CD34 percentages[2,4–6] and Pgp levels, in turn, have been related to levels of MRD.[7] Both Pgp and MRD are major factors of poor prognosis.[6,8–14] Moreover, low CD34 expression is often found in *NPM1* mutated patients[15,16] and since *NPM1* mutation is associated with good risk, this suggests an association between CD34 and prognosis. In a meta-analysis of over 2000 patients Kanda et al concluded that CD34 protein expression as measured with flow should not be considered a marker of poor prognosis.[17] However, more recently, gene expression profiling revealed *CD34* mRNA expression to be an independent prognostic marker in cytogenetically intermediate risk AML patients.[18] Thus, the controversy concerning the prognostic relevance of CD34 either protein or mRNA expression is still ongoing.

We hypothesize that the controversial flow cytometric results could be attributed, at least in part, to differences in study design since prognostic impact was always based on arbitrary cut-off values, like 5%, 10%, and 20% CD34-positivity.[17,19,20] Such an approach could lead to incorrect results, since a relatively small, but highly proliferative diagnosis CD34-positive subpopulation, could survive treatment and grow out to a clear CD34-positive AML relapse.[21] Using a cut-off would classify these AML cases as 'CD34low' which would, in some cases, falsely presume a good prognosis. We use a new definition to investigate the clinical relevance of CD34 protein expression. We discriminate between CD34-negative and CD34-positive AML patients without the a priori use of cut-off values: a CD34-negative AML is defined as having only normal haematopoietic cells present in the CD34+ blast compartment, and a CD34-positive AML includes all other cases. Consequently, in a CD34-negative AML, all leukaemic

cells are present in the CD34-negative blast compartment. This contrasts to a CD34-positive AML, where leukaemic cells are present in both the CD34+ and CD34- blast compartment. In this study we demonstrate the prognostic importance of CD34 by the use of a conceptually new method to evaluate CD34 expression in AML and hereby explain discrepancies in previous studies. We show that the prognostic relevance is not strictly related to known associations such as *NPM1* mutations and we demonstrate that when the CD34+ compartments only contains normal cells this is related to favourable prognosis. These findings are put into the light of the leukaemic stem cell concept and therapy resistance. Lastly, being an independent prognostic parameter, we propose to revisit CD34 as a prognostic factor in AML, especially in good and intermediate risk AML patients.

Patients, Materials and Methods

The Data Supplement provides more detailed information.

Patients and treatment

A total of 706 patients (18-65 years) were included, treated according to HOVON/SAKK clinical trials. Details concerning institutional review boards, informed consent and patient treatment are described in the data supplement. Risk classification was as previously published[12], i.e., good risk patients had t(8;21) and WBC ≤20 x 10^9/l, inv16/t(16;16), or absence of a monosomal karyotype concomitantly with the presence of *CEBPA*^{mut} or *NPM1*^{mut}/*FLT3*^{wt} in CR after the first induction cycle. Poor risk was defined as non-core binding factor (CBF) leukaemia with the presence of either a monosomal karyotype, 3q26 abnormalities or *EVI1* overexpression. All remaining patients were classified as intermediate risk AML patients. Patient characteristics are outlined in Table I.

Immunophenotypic CD34 assessment

Fresh BM samples were used to perform multiparameter immunophenotyping. Detailed information regarding sample preparation and the panel of antibodies is provided in the data supplement and Table SI. To discriminate CD34-negative from CD34-positive patients, the LAP (Leukaemia Associated Phenotype) approach, described previously[12,22], was used. LAPs were defined on both CD34+ and CD34- blasts (Fig 1). In case a LAP was present on the CD34+ blasts, the patient was defined as CD34-positive, since this shows that leukaemic cells are present within the CD34+ compartment. In 645 cases in which LAPs could be determined on blast cells, 127/128 patients defined as CD34-negative, had a CD34 percentage ≤1% (Fig S1B). In case of no LAP and CD34 ≤1% (32/706; 4.5%), patients were assumed as CD34-negative. Cryopreserved samples were used for the cell sorting experiments. Thawed cells were labelled with the appropriate antibodies and prior to molecular analysis subfractions sorted with a FACSAria cell sorter (BD).

TABLE I | Patient characteristics

	No. of CD34-Negative Patients	%	No. of CD34-Positive Patients	%	p-value
Total	158		548		
Sex					.77
Male	83	53	295	54	
Female	75	47	253	46	
Age, median	50		51		.17
Range	19-63		18-65		
WBC at diagnosis x10^9/L					.001
Median	21.1		8.6		
Range	0.5-303.3		0.4-322.0		
FAB (%)					<.001
M0	3	2	59	11	
M1	27	17	102	19	
M2	38	24	147	27	
M4	22	14	80	15	
M5	48	30	46	8	
M6	4	3	16	3	
M7	1	1	3	1	
RAEB	4	3	36	7	
RAEB-t	7	4	34	6	
Not classified	4	3	25	5	
AML type					.014
De Novo	139	88	424	77	
Secondary	8	5	54	10	
MDS	11	7	70	13	
Core-binding Factor-AML					<.001
No	133	84	397	72	
Yes	0	0	68	12	
ND	25	16	83	15	
FLT3/NPM1 status					<.001
FLT3^{wt} / NPM1^{wt}	38	24	328	59	
FLT3^{wt} / NPM1^{mut}	65	41	33	6	
FLT3^{ITD} / NPM1^{wt}	6	4	51	9	
FLT3^{ITD} / NPM1^{mut}	23	15	42	8	
Missing	26	16	94	17	
Mutated CEBPα					.10
Neg	128	81	403	74	
Pos	2	1	20	4	
ND	28	18	125	23	
EVI1					.002
Neg	111	70	354	65	
Pos	7	4	73	13	
ND	40	25	121	22	
Molecular/cytogenetic risk group					<.001
Good	29	18	89	16	
Intermediate	117	74	339	62	
Poor	12	8	120	22	
CR reached					.032
Never CR	19	12	121	22	
After cycle 1	104	66	319	58	
After cycle 2	29	18	81	15	
Later	6	4	27	5	
Last consolidation therapy					<.001
None	45	28	247	45	
3rd cycle	27	17	85	16	
Autologous SCT	34	22	60	11	
Allogeneic SCT	52	33	156	28	

ND, not determined. Core-binding factor AML defined as translocation[8;21]/inv[16] or t[16;16]; CR, complete remission; FAB, French-American-British classification; SCT, stem cell transplantation. RAEB, refractory anaemia with excess blasts; RAEB-t, RAEB in transformation; WBC, white blood cell.

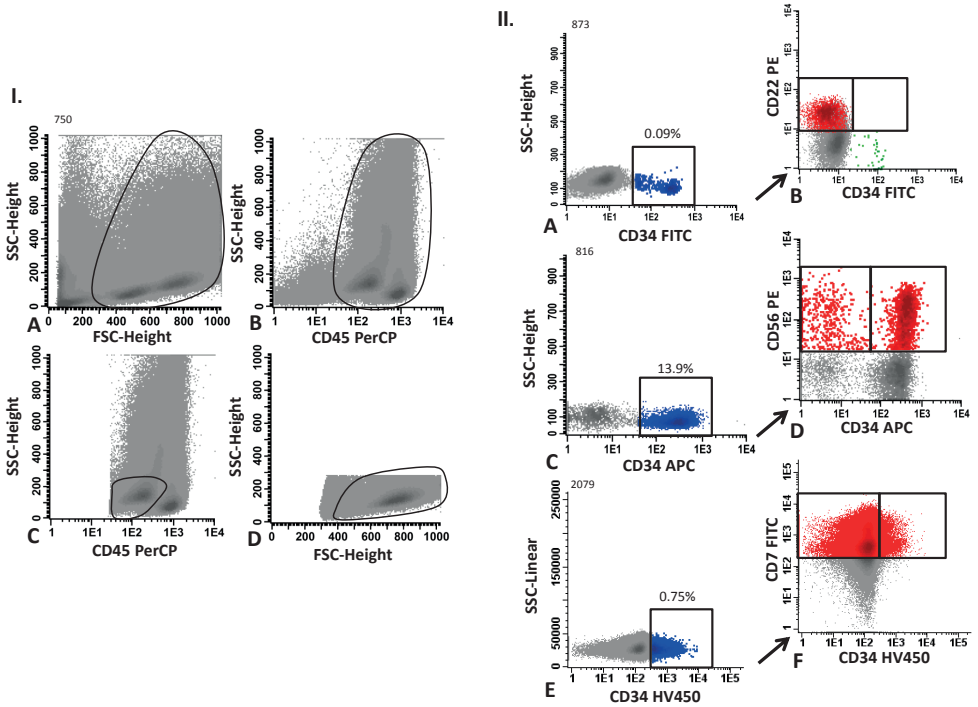

FIGURE 1 | Flow cytometric approach to identify a leukaemia as CD34-negative or CD34-positive.
I. Gating of CD45dim blast cells
After lysis, bone marrow cells were washed and labelled with the appropriate antibodies (Table SI). Dead cells and remaining erythrocytes were excluded in a FSC/SSC plot (A). CD45low/negative cells were excluded to identify white blood cells (B). Blasts were identified based on CD45dim and low SSC properties (C). To define a homogenous population of cells, blasts cells were also gated in a FSC/SSC plot (D).
II. Identification of CD34-negative or CD34-positive AMLs
Blasts were identified as shown in Fig 1.I and subsequently CD34 cells were gated in this fraction. A,B. CD34-negative patient (nr 873) for which CD34+ cells (blue cells) are 0.09% of the total number of WBC (A). Aberrant expression of CD22 (B, cells in red) was present on the CD34- blasts, implying that these (non-lymphocytic) cells are neoplastic. The small population of CD34+ cells (B, cells in green) lack aberrant marker expression, suggesting that these cells are normal haematopoietic cells. C,D. A CD34-positive AML (nr 816) is shown with a CD34+ percentage of 13.9%. Aberrant expression of CD56 (red) was present on both CD34- and CD34+ blasts (D) and therefore the patient was defined as CD34-positive. E,F. CD34-positive patient (nr 2079) with a low (<1%) frequency of CD34+ cells. Aberrant expression of CD7 was present on a large fraction of CD34- cells and it was also found on (almost) all CD34+ cells (F), implying that neoplastic cells were also present in this small CD34+ population.

Molecular analysis

Cryo-preserved cells were used to determine mutation statuses from sorted cell populations and DNA was isolated using the Qiagen Allprep kit (Qiagen Benelux B.V., Venlo, The Netherlands). Genomic DNA from sorted cell populations was analysed by PCR to determine the presence of *FLT3*^{itd} and/or *NPM1* mutation in the different subpopulations.

MRD assessment

Flow cytometric MRD assessment was performed as described previously (details in Supplementary).[12] MRD results after the second chemotherapy cycle were available in 39% (272/706) of all patients. Importantly, MRD results were only assessed in CR patients.

Statistical analysis

Categorical variables were compared using the chi-square test. The non-parametric Mann-Whitney U test was used for continuous variables. Cumulative Incidence of Relapse (CIR) was calculated from date of CR to date of relapse and death was hereby included as a competing event using the Fine and Gray model.[23] Since date of CR was missing in 24/566 CR patients (Table I) CIR curves were generated for 542 patients (Fig 3). OS was measured from diagnosis to date of death. Patients without a defined event were censored at the date of last contact. Event free survival (EFS) refers to the time from diagnosis until the event. An event was hereby defined as 1) date of response evaluation after the last induction cycle if CR had not been achieved 2) date of relapse or 3) date of death, whichever came first. For survival analysis including MRD, EFS and OS are measured from date of sampling after the second cycle until above-mentioned endpoints. Kaplan-Meier curves were generated and survival between groups was compared using log-rank tests. To compare EFS for the different AML risk categories a forest plot of hazard ratios (HR) including 95% confidence intervals (CI) was generated and p-values were calculated using the log-rank test. A trend test for the HRs in the three risk groups was performed by including CD34 status, the risk group as well as their interaction term in a Cox regression model.

Results

In a CD34-negative AML all CD34+ blasts are non-neoplastic cells

We defined a CD34-negative AML as having no aberrant immunophenotypes, and/or cytogenetic and molecular abnormalities in the CD34+ fraction. Consequently, all leukaemic cells are in the CD34- compartment. Fig 1(II.A-B) shows a CD34-negative sample with aberrant CD22 expression. As shown in the CD22/CD34 plot (B), the small population (0.09%) of CD34+ blasts lacks CD22 expression, while a large fraction of CD34- blasts does have aberrant CD22 expression. Fig 2(I) shows another sample with CD34<0.1% and no aberrant immunophenotypes present on the CD34+ blasts. This implies that all CD34+ cells are non-neoplastic cells (for reasons of simplicity, further referred to as normal haematopoietic cells). The CD34- blasts in this case are considered neoplastic since different LAPs were present on these cells (#1719, Table SII). This AML is characterized by a *FLT3*^{itd} mutation. Molecular subfraction analyses revealed that the mutation is only present in the CD34- blasts (Fig 2I.C) and not in the CD34+ cells (Fig 2I.B). Table SII shows an overview of 9 AML samples, which, based on flow cytometry qualified for CD34-negativity. In these samples normal character of CD34+ cells could be confirmed by PCR analysis.

In a CD34-positive AML both CD34- and CD34+ blast compartments contain neoplastic cells

In a CD34-positive AML by definition the CD34+ fraction contains leukaemic cells. This is represented by the presence of (an) aberrant immunophenotype(s) on the blast population. Thus, in case one or more LAPs were present on at least part of the CD34+ blasts, the patient was defined as CD34-positive (Fig 1II.C-F). Fig 2(II) shows a CD34-positive AML characterized by an *NPM1* mutation. PCR analysis of cell sorted subfractions revealed that the *NPM1* mutation was present in both the CD34- and CD34+ compartment, implying that both compartments contain leukaemic cells (Fig 2II.A-C). Molecular subfraction analyses were performed in 9, flow cytometry defined CD34-positive cases (Table SII). In the absence of LAPs, two patients were defined positive only based on a CD34 percentage >1% (Table SII; #1263, #2005). In all 9 cases PCR confirmed the presence of leukaemic cells in CD34+ and also in CD34- compartments.

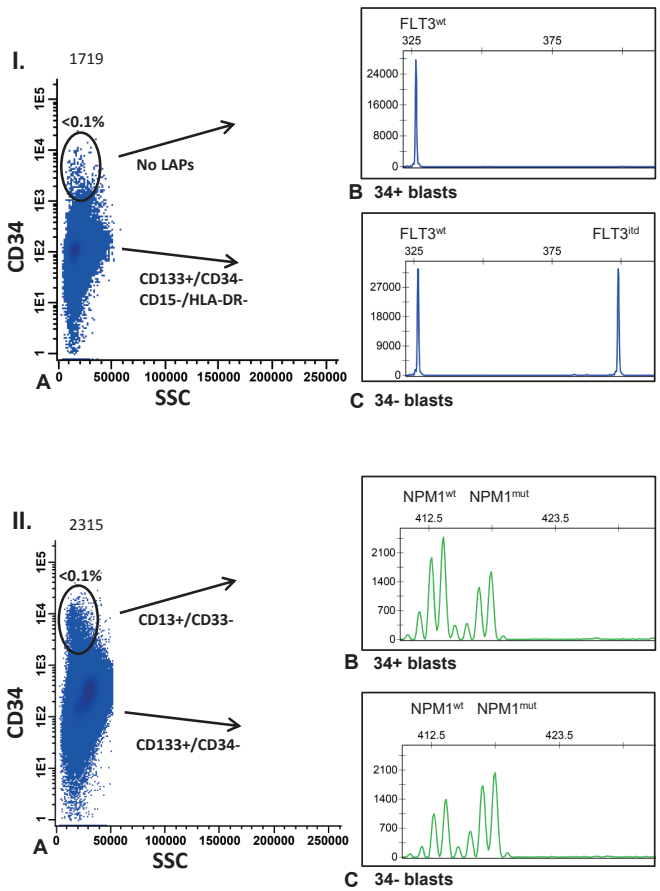

FIGURE 2 | CD34+blasts are normal in a CD34-negative AML and neoplastic in a CD34-positive AML. I. Gating/sorting strategy for patient 1719.

Blast cells were gated as shown in Fig 1.I and CD34+ cells were gated within this fraction of cells (A). Based on flow cytometry this patient was qualified as CD34-negative since no LAPs were present on the CD34+

cells, but do so on CD34- cells (CD133+/CD34-; CD15-/HLA-DR-), while the frequency of CD34+ cells was <1% (Table SII). The neoplastic blasts in this patient were characterized by the presence of a *FLT3*^{itd} mutation (heterozygous: not shown). After cell sorting, molecular analyses showed that the CD34+ blasts lack the *FLT3*^{itd} mutation and are therefore presumed normal (B), while the CD34- blasts were neoplastic since these harbored a *FLT3*^{itd} (C).

II. Gating/sorting strategy for patient 2315.

CD34+ cells (A) were gated as described above for patient 1719. Based on flow cytometry this patient was qualified as CD34-positive since an aberrant immunophenotype (CD13+/CD33-, Table SII) was present on part of the CD34+ blasts. The leukaemic blasts in this patient were characterized by the presence of the *NPM1* mutation (not shown). After cell sorting, molecular analyses showed that the *NPM1* mutation was present in both the CD34+ and the CD34- blasts and thus both these blast fractions were neoplastic.

Association of CD34 status and clinical features

In total 158/706 patients (22.4%) were categorized as CD34-negative and 548 patients (77.6%) as CD34-positive (Fig SI.A). CD34-negativity appeared to be associated with a very low CD34 percentage: median CD34 as a percentage of total WBC was 0.10 (0.00%-1.04%; only 1/158 patients had CD34 expression >1.0%, i.e. 1.04%). This implies that an AML with CD34 percentage >1% is CD34-positive (Fig SI.A). The CD34-positive status was found in a large range of CD34 percentages: median was 23.1 (range 0.05%-95.2%; 44/548 patients had CD34%≤1.0). This implies that CD34 percentage ≤1% is not fully associated with CD34-negativity. Furthermore, in CD34-negative patients the frequency of *NPM1*^{mut}*/FLT3*^{wt} was 49% (65/132 evaluable patients, Table I). In CD34-positive patients this was 7% (33/454). However, as shown in Table I, the presence of CD34-negative patients is not limited to the good risk category since 82% of CD34-negative patients belong to the intermediate or poor risk group, because of additional poor prognostic cytogenetic or molecular abnormalities, such as *FLT3*^{itd}. Also, as shown in Table I, acute monoblastic and acute monocytic leukaemia (FAB M5) occur more frequently in CD34-negative as compared to CD34-positive patients (30% vs 8% respectively, p<0.001). Additionally, CD34-negative patients had a higher WBC count at diagnosis (median 21.1x10^9/L, range 0.5-303.3) as compared to CD34-positive patients (median 8.6x10^9/L, range 0.4-322.0; p=0.001).

Prognostic relevance of CD34 expression

After having established the definition of CD34-positivity and negativity, the prognostic relevance of CD34 was investigated and compared with cut-offs used in literature, i.e. 5%, 10% and 20%, and which did not include assessment of neoplastic character.[17,19,20] The use of our new definition for CD34-positivity in AML revealed a difference in response to treatment: in the CD34-negative cohort 12% (19/158) never achieved CR (NCR) as compared to 22% (121/548) of the CD34-positive patients (p=0.005). Although CD34-positivity appeared not to be an independent marker for achieving CR after adjusting for other clinical relevant variables (HR 0.88, 95% CI 0.68-1.14), differences in achieving CR became less pronounced when using increasing cut-off values: when using a 5% and 10% cut-off differences were as follows: CD34≤5% 14% NCR (39/273) vs CD34>5% 23% NCR (101/433, p=0.003) and for the

10% cut-off it was 16% NCR in CD34≤10% vs 23% NCR in CD34>10%, p=0.01). Differences were no longer significant with a 20% cut-off (18% vs 23% NCR, p=0.09). A striking difference was seen with our new approach for CIR, since relapse incidence at 4-years was 28% (95% CI 20%-36%) for CD34-negative patients and 50% (95% CI 40%-50%) for CD34-positive patients (p<0.001) (Fig 3A).

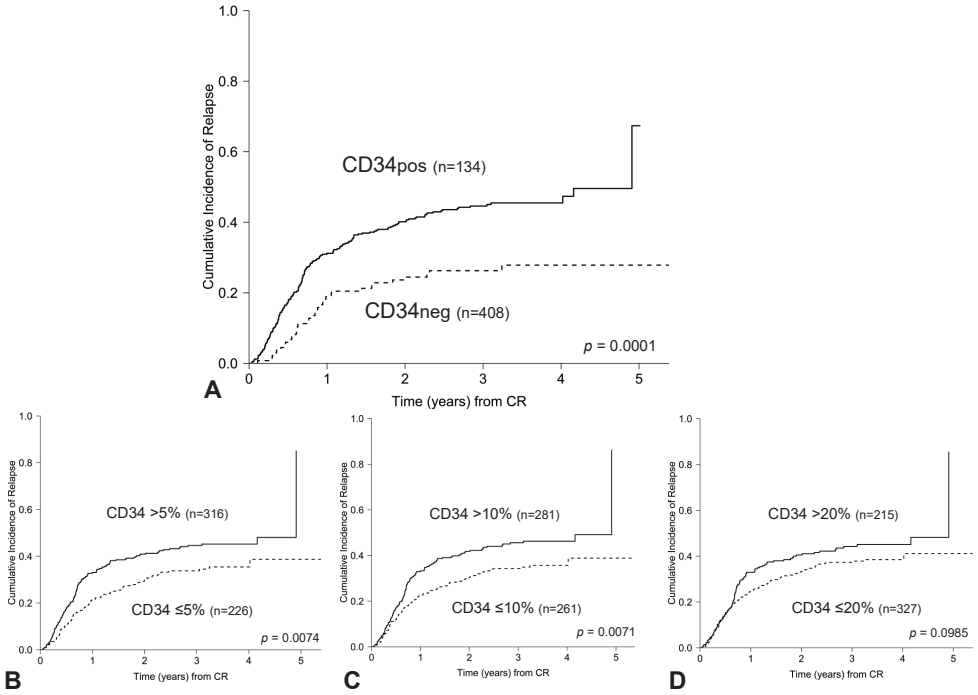

FIGURE 3 | Cumulative Incidence of Relapse for CD34-negative and CD34-positive AML patients
Cumulative incidence of relapse, with death as a competing event, whereby different approaches are used to estimate the prognostic relevance of CD34: 1) our new approach to define CD34-positive and negative patients without structural use of a cut-off value (A); 2) using a 5% cut-off value (B); 3) using a 10% cut-off value (C) and 4) using a 20% cut-off value (D), all to define CD34 low and CD34 high patients. The new definition of CD34 shows the strongest prognostic impact. Fig S2 and Fig S3 shows the same results for EFS and OS, respectively.

Similar differences were also found for OS (Fig S3): 4-yr OS of CD34-negative patients was 62% vs 39% for CD34-positive patients (p<0.001). Subsequently, we compared our new CD34 approach with the classical cut-off approach. The prognostic impact of CD34% decreases when comparing our definition for CD34-positivity in AML with above-mentioned cut-off levels (Fig 3B-D, Fig S2B-D and Fig S3B-D). Our hypothesis was that (remaining) prognostic impact seen with classical cut-offs is largely due to the presence of relatively favorable prognostic CD34-negative AML patients as present in all the low CD34 groups (<5%, <10%, <20%). To verify if our defined CD34-negative patients indeed account for the remaining prognostic differences found when using a cut-off value (Fig 3B-D, Fig S2B-D and Fig S3B-D), the prognostic impact of CD34% was also assessed in only the CD34-positive patients (n=548 in total of which n=408 in CR); thus omitting the CD34-negative group. The prognostic impact (CIR) of CD34 was

completely lost in this group of true CD34-positive patients (Fig 4A-C). Similar results were obtained for OS (Fig 4D-F). These results demonstrate that the prognostic impact of previously used cut-off approaches entirely depend on the inclusion of sufficient numbers of truly CD34-negative patients, as we now define by complete absence of neoplastic CD34+ cells. Finally, multivariate analysis showed that the new definition of CD34, defined at time of diagnosis, is an independent prognostic factor (EFS HR 1.66, 95% CI 1.19-2.31; RFS HR 1.81, 95% CI 1.15-2.85; OS HR 1.57, 95% CI 1.08-2.29; Table SIII, EFS not shown).

The prognostic relevance of CD34 in different subsets of AML patients

Since risk adapted therapy is nowadays common in AML, it is also of importance to check the possible additional value of CD34 in different subsets of AML patients. It appeared that CD34 could significantly add to predicting outcome in good risk AML patients: 4-yr EFS of 29 CD34-negative patients was 84% (standard error [SE] 7%) as compared to 50% (SE 6%) for 89 CD34-positive patients (p=0.002). This implies that CD34-positive patients in the good risk group have a more intermediate prognosis as compared to good risk CD34-negative patients who actually have a very good prognosis. Also in the intermediate risk group 4-yr EFS is significantly better in CD34-negative patients as compared to CD34-positive patients (48% [SE 5%] vs 32% [SE 3%] respectively, p=0.001), reclassifying intermediate risk CD34-positive patients as poor risk. No significant difference was found for the poor risk group. Fig 5 shows differences in HRs for EFS based on CD34 status for these different AML risk subgroups. Moreover, since there is a known association between CD34 and *NPM1*^{mut}*/FLT3*^{itd} mutant status, we divided the AML patient group into 4 groups based on *FLT3/NPM1* genotypes. Although no difference was found in the intermediate risk *NPM1*^{mut}*/FLT3*^{wt} and the poor risk *NPM1*^{wt}*/FLT3*^{wt} patient groups, CD34-negative patients performed better than CD34-positive patients in all other risk groups (Table SIV).

Association between CD34 and MRD

At present, it is well established that MRD assessment is an important factor in predicting risk of relapse and/or death.[12,22,24–27] In upcoming clinical studies, MRD assessment will therefore be included in risk group stratification (e.g. HOVON132). Hence, we estimated the role of CD34 in different risk groups, also taking into account MRD results. MRD results were available in a group of 272 patients with known CD34 status, including 70 (25.7%) CD34-negative and 202 (74.3%) CD34-positive patients. In the CD34-negative group only 11.4% (8/70) of CD34-negative patients appeared to be MRD-positive as defined after the second cycle of chemotherapy treatment as compared to 29.7% (60/202) of CD34-positive patients (p<0.005). Overall, these data indicate that CD34-positive patients at time of diagnosis are 3.3-times more likely to be MRD-positive after induction therapy than CD34-negative patients (95% CI 1.5-7.3, p=0.003). Since MRD is thought to represent the resultant of many resistance mechanisms, we included MRD status in the multivariate analysis. This cohort is significantly smaller (n=235), however CD34 status remains an independent predictor for worse outcome (RFS HR 1.96, 95% CI 1.06-3.63; OS HR 2.06, 95% CI 1.10-3.86). Fig S4 shows that MRD further adds to the prognostic impact of CD34, especially in good and intermediate risk patients (Fig S4I.C and II.C).

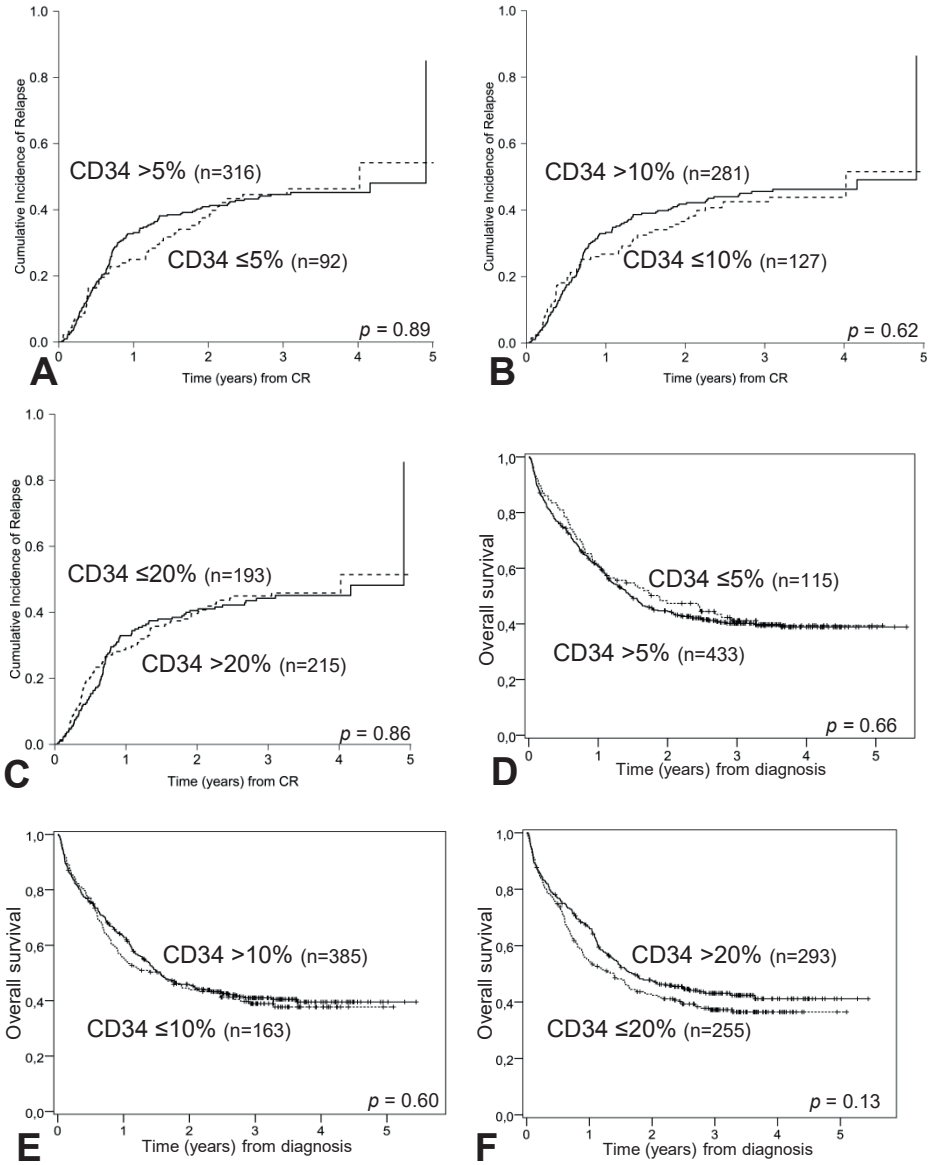

FIGURE 4 | Prognostic value of CD34 at diagnosis in CD34-positive patients.
This figure shows three CIR and three Kaplan-Meier OS curves whereby different cut-offs are used to estimate the prognostic relevance of CD34 solely in the group of CD34-positive patients, thus after excluding CD34-negative patients: A) using a 5% cut-off value, (B) using a 10% cut-off value, and (C) using a 20% cut-off value to define CD34low and CD34high patients. (D-F) shows the same results for OS. Leaving out the CD34-negative group from the analyses thus almost completely abrogated the prognostic impact of CD34.

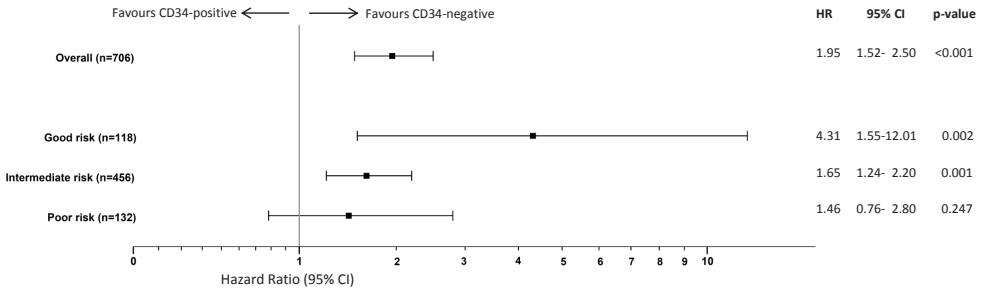

FIGURE 5 | Forest plot of hazard ratios for event free survival in different risk categories.
Forest plot of hazard ratios for EFS rates for 118 good risk, 456 intermediate risk and 132 poor risk AML patients. For proper comparison, the hazard ratio of the overall patient cohort (n=706) is shown in the upper part of the plot. Horizontal lines represent 95% confidence intervals (CI). There was no evidence for a trend of decreasing HRs with increasing risk (p=0.203).

Discussion

We reconsidered the role of CD34 expression in outcome of AML patients and revealed an explanation for the contradictory results demonstrated previously.[28–31] We established a powerful new definition of CD34-positivity, whereby we think that incorporation of this novel definition into existing risk profiles is warranted.

To summarize our observations:

1. CD34 has prognostic value in the present group of 706 patients by applying cut-offs used in literature. However, upon increasing cut-off levels the prognostic value was gradually lost.
2. CD34-negative patients do not have neoplastic CD34+ cells. These AML patients were defined by the absence of aberrant immunophenotypes, which was in some cases confirmed by molecular analysis of sorted CD34+ fractions.
3. All patients with CD34>1.04% carried neoplastic CD34+ cells and were referred to as CD34-positive.
4. In the total CD34-positive group there were AML patients with neoplastic CD34+ cells present, but with CD34 percentage <1% (8.0% of total CD34-positive group).
5. Leaving out the CD34-negative group from the survival analyses completely abrogated the prognostic impact of CD34 expression in the remaining CD34-positive group when applying cut-offs used in literature.
6. When applying our new definition, CD34 has strong prognostic impact in several risk subgroups.
7. MRD frequency assessed after induction therapy was higher in CD34-positive patients. Moreover, the combination of CD34 status at diagnosis and MRD as follow-up parameter holds clear promises for prognostics.

CD34 has been defined as an important prognostic marker for adverse outcome previously.[28,29,32–35] However, others disproved these observations.[30,31,36–40] Based on these contradictory results CD34 expression was considered not to be beneficial as prognostic marker.[17] The controversial results could be (partly) attributed to differences in definition of CD34-positivity, since prognostic impact was based on different cut-off values. To avoid such a methodological pitfall, we used a new definition, without the a priori use of a cut-off. This definition uses presence or absence of neoplastic CD34+ cells and this now appears to be a powerful predictor for CIR, EFS and OS in the entire group of AML patients. Therefore, this new definition not only explains conflicting results published in the past, but also reveals that this independent prognostic marker should be incorporated into AML risk stratification.

Previously we defined CD34-negativity mainly based on functional properties, without considering impact on prognosis.[5] Here, we have used immunophenotypic aberrancies as they are routinely assessed at diagnosis for diagnostics and/or to enable later MRD assessment.[12,22] Also in the absence of immunophenotypic and/or molecular aberrancies, the AML can relatively safely be diagnosed as CD34-positive when CD34 is >1%. Importantly, a small group of patients (44/548; 8.0%) had CD34 ≤1% yet harbored neoplastic CD34+ cells (Fig S1). This may be of importance to predict clonal changes during therapy, since we previously found that relapse populations may be traced back as very small CD34+ subpopulations at diagnosis.[21] A patient with ≤1% CD34 can thus not a priori be defined as CD34-negative and LAP assessment is thus necessary. In case all samples without immunophenotypic aberrancies and with ≤1% CD34 would be defined as CD34-negative, false-negativity may then amount to 22% (44/(44+157) (Fig S1A). Importantly, there is a small group of patients with CD34 ≤1% but no immunophenotypic aberrancies present in our dataset (32/61 patients with no LAP). There thus is a chance that some of these 32 cases (estimated 22%) have neoplastic CD34+ cells and thus are false-negative. The probability of false-negativity in this group is however small: estimated 7/32 (22%) patients. In the total population this corresponds to <1% (namely 7 out of 706 patients). Moreover, in our current 8-colour panel more antibody combinations are included, making the probability of false-negativity in a future cohort even smaller. To retrospectively validate the CD34 status in the small group of patients with no LAP, functional assays could be used, like aldehyde dehydrogenase (ALDH), since it has been established that normal haematopoietic cells have a higher ALDH activity as compared to leukaemic cells.[41,42] Importantly, no differences in cytogenetic/molecular risk profiles or other clinical characteristics were detected between patients with no LAP and patients with (a) LAP(s), and, moreover, survival differences between CD34-negative and CD34-positive patients were not different when only including patients with a LAP (n=645) as compared to also including patients without a LAP (n=706, data not shown).The prognostic impact of CD34 was almost entirely due to the presence of CD34-negative patients (Fig 4). This explains why increasing cut-off levels result in disappearance of prognostic value: increasing numbers of poor prognosis CD34-positive patients will enter the group below the cut-off level, thereby annihilating survival differences. For example when using a 10% cut-off, increasing numbers of poor prognosis CD34-positive patients are included in the group below the 10% cut-off level which results in poorer prognosis of that subset. In the past the following discrepancies in AML patient cohorts likely have contributed to the varying results published concerning the prognostic relevance. Firstly,

the size of the truly CD34-negative group may have varied between studies, especially when relatively small patient groups or non-randomly selected patient groups were studied. Secondly, a relatively high proportion of good prognosis patients with the t(8;21), inv(16) or *CEBPA* double mutant molecular profile, which are known to be CD34-positive, will "pollute" the CD34+ poor prognosis group with these good prognosis patients.[43] This may outweigh the differences that exist with the good prognostic CD34-negative group. Extensive immunophenotyping is common in AML diagnostics and this allows the detection of aberrant immunophenotypes on CD34+ cells and thereby their neoplastic character. Therefore, implementation of CD34 in risk assessment at diagnosis is relatively easy and far more specific compared to arbitrary cut-offs used previously.

In literature *NPM1* mutated AML patients have lower CD34 expression compared to other groups.[15,16] We also have found a clear association between CD34-negativity and the *FLT3*^{wt}/*NPM1*^{mut} profile. It also appeared that CD34 status enables distinction of a CD34-negative group with a very good prognosis and a CD34-positive group with a more intermediate prognosis within *NPM1*^{mut} AML (Table SIV). These results are consistent with recent studies demonstrating CD34 as a predictor of prognosis in *NPM1*-mutated patients.[19,20] Altogether, this knowledge should be incorporated into future risk-adapted therapy in this subset of good risk AML patients.

CD34 status at diagnosis was associated with MRD in our large AML patient cohort. In this respect, it would also be of interest to investigate the presence of leukaemia initiating cells, since MRD is supposed to be caused by the outgrowth of such cells.[44] Such leukaemia initiating capacity can be accommodated in both the CD34+ and CD34- blast compartment[15,44–47], however, it has been shown that, probably depending on the mouse model used, a subset of CD34+ blast (namely CD34+CD38-), has the highest engraftment potential in mice.[48,49] This suggests that (this fraction of) blasts, is either more therapy resistant or otherwise more resistant to apoptosis.[50] Moreover, CD34+CD38- cells were shown *in vitro* and *in vivo* to be most therapy resistant.[48,51] These results suggest that the absence of CD34+(CD38-) cells may contribute to the good prognosis in CD34-negative patients since these completely lack neoplastic CD34+(CD38-) cells. It also implies that in CD34-positive patients, the frequency of CD34+CD38- leukaemia initiating cells could be of prognostic relevance.[52] In line with that, in a cohort of 88 AML patients, of the different CD34/CD38 defined cell fractions (CD34+CD38-/CD34+CD38+/CD34-) it was only the CD34+CD38- compartment that, both at diagnosis and follow-up, had prognostic impact.[44] Therefore, risk estimation in our CD34-positive patients could, theoretically, be further refined taking into account not only MRD status, but also the fraction of neoplastic CD34+CD38- cells. Future studies should be carried out to confirm these findings.

In summary, we think we have solved the long lasting mystery of contradictory results in literature as to prognostic impact of CD34 expression in AML that until now has prevented incorporation of this relatively simple flowcytometric parameter in AML risk-stratification. We showed that the absence of neoplastic CD34+ cells determines the prognostic impact of CD34 expression in AML. We propose a prognostic model whereby CD34 status is combined with current well defined risk factors at base line as well as on treatment factors including MRD cell frequency.

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Supporting information

Patients and treatment

A total of 706 AML patients, treated according to the HOVON42a (n=438, 2001-2008), HOVON92 (n=95, 2008-2010) or HOVON102 (n=173, 2010-2013) multicenter clinical trials, were included ([HOVON/SAKK AML] Dutch-Belgian Hemato-Oncology Cooperative Group/Swiss Group for Clinical Cancer Research Acute Myeloid Leukemia). All patients had a cytopathologically confirmed diagnosis of AML according to the WHO classification (excluding acute promyelocytic leukemia) or a diagnosis of refractory anaemia with excess of blasts and IPSS score ≥1.5. The HOVON/SAKK42a, 92 and 102 studies were reviewed and approved by an institutional review board (METc) of the Erasmus MC Rotterdam for the total study (number 2000-220 for HOVON 42a, and 2008/216 for HOVON92, and 2009/293 for HOVON102). In addition, the VU University Medical Center review board approved both studies with METc number 2001/50 (LUV), 2008/292 (LUV) and 2010/56 (LUV), respectively. Patients provided their written informed consent to participate. Detailed information concerning inclusion and exclusion criteria and information concerning treatment protocols can be found on the HOVON website (www.hovon.nl). In brief, the majority of patients received two cycles of induction therapy followed by either a third cycle of chemotherapy, or autologous or allogeneic stem cell transplantation. In all three studies the standard arm of the first induction cycle consisted of idarubicin and cytarabine. Standard arm of the second cycle contained amsacrine and cytarabine and in case a third cycle of chemotherapy was given for consolidation therapy, this consisted of mitoxantrone and etoposide. Moreover, in these treatment protocols patients were randomized to either the standard treatment arm or an experimental treatment arm. The experimental arm in the HOVON42a consisted of standard induction therapy as described above combined with granulocyte-colony-stimulating factor (G-CSF). Patients randomized to the experimental arm of the HOVON92 received standard induction therapy combined with laromustine, an alkylating agent and in the HOVON102 standard induction therapy was combined with clofarabine. The HOVON92 study was stopped early due to alleged toxicity of the experimental drug. For the HOVON102 study is it still unknown which patients were treated with the experimental drug. Since, in our patient cohort, no clinically relevant differences could be found between the different treatment arms in the HOVON42a and HOVON92, data were pooled.

Immunophenotypic CD34 assessment

At time of diagnosis erythrocyte-lysed (Pharm lyse, Becton and Dickinson [BD]) BM samples were used to perform multiparameter immunophenotyping by flow cytometry. After lysis cells were washed with phosphate buffered saline/0.1% human serum albumin and labelled with the appropriate antibodies (Table SI). Cells were incubated with the antibodies for 15 minutes at room temperature in the dark. Subsequently, cells were washed to remove unlabelled antibodies. Per tube $2x10^6$ cells were labelled and a minimum of 50,000 WBCs were acquired. Thereafter measurements were performed using a FACSCalibur and FACSCanto from BD (New Jersey, USA). Samples were considered eligible for CD34 analysis when a cluster of

CD34+/SSClow cells was present. LAPs on both the CD34+ and CD34- blasts were detected as described previously.[1,2] The LAP approach encompasses the detection of combinations of cell surface markers that are not, or in low frequencies, present on normal BM cells. First, leukaemic blasts were gated based on CD45dim and low side scatter properties (SSClow) (Fig 1.I). After that, LAPs were defined using a primitive marker (CD34 and/or CD117 and/or CD133) in combination with a myeloid marker and an aberrantly expressed cell surface marker (i.e. CD2, CD7, CD11b, CD15, CD19, CD22 or CD56). Additionally, in very few cases were LAPs could not be well assessed, C-type lectin-like molecule-1 (CLL-1) and CD33 were used to investigate aberrant expression on the subset of CD34+CD38- cells. Importantly, LAPs which were present on <10% of blasts cells were also included since also small leukaemic subpopulations are of importance in the determination of CD34 status. The HOVON42a and 92 studies were measured with a 4-colour antibody panel, and the samples of the HOVON102 study were measured with a 6- and 8-colour antibody panel (Table SI). All immunophenotypic analyses were performed using both CellQuest and Infinicyt™ software (Cytognos, Spain).

Molecular analysis

PCR was applied to determine the presence of *FLT3*^{itd} and/or *NPM1* mutations in the different subpopulations. Mutations in *NPM1* (exon12) were analysed with the following primers: forward: 5′-TTAACTCTCTGGTGGTAGAATGA-3′; reverse: 5′-CTGACCACCGCTACTATGT-3′, located in intron 11 and exon 12 of the *NPM1* genomic DNA, respectively. *FLT3* was amplified using the primers covering the entire transmembrane and juxtamembrane domains of *FLT3*. Subsequently, a tetrachlorofluorescein phosphoramidite-labelled forward primer (Biolegio, Nijmegen, The Netherlands) was used for the fragment analysis. For both *FLT3* and *NPM1* analysis, lymphocytes served as an internal negative control and the bulk of the AML blasts served as an internal positive control.

MRD assessment

To define minimal residual disease (MRD) levels during follow-up, LAPs were defined in the diagnosis material as described above. In follow-up samples, about $2x10^6$ cells were labelled in a volume of 50µl with the most optimal LAPs that were defined at diagnosis. MRD levels were only assessed in morphologic remission samples. Gating of blasts was performed as described in the paragraph above and as illustrated in Fig 1.I. Analysis of LAP positive cells was performed on blasts possessing the primitive marker that was used at diagnosis (CD34, CD117 or CD133). MRD levels were defined as the percentage of LAP positive cells within the population of white blood cells. This percentage was multiplied by a correction factor: 100%/percentage of LAP positive blasts at diagnosis. A thus defined final percentage of 0.1% was used to define MRD negative and MRD positive samples.[1]

Statistical analysis

Statistical analyses were performed using the SPSS 20.0 software. The calculation of hazard ratios and its associated 95% confidence interval (CI) for overall survival (OS) and relapse-free survival (RFS) were done using Cox-regression univariate and multivariate analyses. All variables of known clinical importance were included (full data for OS was available in 566 patients and since RFS was assessed in CR patients only, multivariate analysis for RFS was possible in 455 patients), and the analysis was performed using the 'Enter' method. Cumulative Incidence of Relapse (CIR) curves were calculated with R statistics version 2.6.2 and death was herein included as a competing event. P-values were considered significant below 0.05.

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TABLE S1 | Antibody panels

4- Colour antibody panel

Tube	FITC	PE	PerCP	APC
1	PBS	PBS	CD45 (2D1, BD)	PBS
2	CD34 (8G12, BD)	CD22 (S-HCL-1, BD)	CD45	CD117 (104D2, DC)
3	CD15 (MMA, BD)	CD13 (L138, BD)	CD45	CD14 (MoP9, BD)
4	HLA-DR (L243, BD)	CD33 (P67.6, BD)	CD45	CD11b (D12, BD)
5	CD2 (MT910, DC)	CD56 (My31, BD)	CD45	CD7 (M-T701, BD)
6	CD36 (CLB-IVC7, Sanquin)	CD133 (AC133/1, MB)	CD45	CD19 (SJ25C1, BD)

6-Colour antibody panel

Tube	FITC	PE	PerCP	PC7	APC	APC-H7
1	PBS	PBS	CD45 (2D1, BD)	CD34 (581, BC)	PBS	PBS
2	CD2 (MT910, DC)	CD7 (M-T701, BD)	CD45	CD34	CD13 (WM15, Pharmigen)	HLA-DR (L243, BD)
3	CD36 (CLB-IVC7, Sanquin)	CD133 (AC133/1, MB)	CD45	CD34	CD22 (S-HCL-1, BD)	CD19 (SJ25C1, BD)
4	CD15 (MMA, BD)	CD33 (P67.6, BD)	CD45	CD34	CD11b (D12, BD)	CD14 (MoP9, BD)
5	CD13 (WM-47, DC)	CD56 (My31, BD)	CD45	CD34	CD117 (104D2, DC)	HLA-DR

8-Colour antibody panel

Tube	FITC	PE	PerCP-CY5.5	PC7	APC	APC-H7
1	PBS	PBS	CD13 (WM15, BD)	PBS	PBS	PBS
2	CD44 (J173, BC)	CLL-1 (687317, R&D)	CD13	CD56 (N901, BC)	CD38 (HB7, BD)	HLA-DR (L243, BD)
3	CD7 (M-T701, BD)	TIM-3 (344823, R&D)	CD13	CD117 (104D2D1, BC)	CD38	CD19 (SJ25C1, BD)
4	CD2 (MT910, DC)	CD133 (AC133/1, MB)	CD13	CD117	CD38	CD19
5	CD36 (CLB-IVC7, Sanquin)	CD123 (9F5, BD)	CD13	CD33 (D3HI60.251, BC)	CD38	CD14 (MoP9, BD)
6	CD11b (Bear1, BC)	CD96 (6F9, BD)	CD13	CD117	CD38	CD14
7	CD15 (MMA, BD)	CD22 (S-HCL-1, BD)	CD13	CD117	CD38	HLA-DR

To increase the sensitivity and specificity of a LAP a second labeling was performed in the 4-and 6 colour panels, most often including CD34. This second labeling often included a label switch of one or more antibodies, dependent of the LAP(s) present in the patient. Associated clones are given in brackets. BD, Becton, Dickinson and Company; BC, Beckman Coulter; DC, DakoCytomation; MB, Miltenyi Biotec; R&D, R&D systems.

TABLE SII | Immunophenotypic and molecular characteristics of CD34-negative and CD34-positive sorted AML samples

Patient nr	CD34%	Molecular status of blasts cells	LAPS on CD34+ blasts	LAPS on CD34- blasts	Molecular status of CD34+ blasts	Molecular status of CD34- blasts
CD34neg						
508	1.1	$NPM1^{mut}$	None	CD33+/CD13-, CD56	$NPM1^{wt}$	$NPM1^{mut}$
1027	0.1	$FLT3^{ITD}$	None	None	$FLT3^{wt}$	$FLT3^{ITD}$
1073	<0.1	$FLT3^{ITD}$	None	CD56 , CD15-/HLA-DR-	$FLT3^{wt}$	$FLT3^{ITD}$
1103	<0.1	$NPM1^{mut}$	None	None	$NPM1^{wt}$	$NPM1^{mut}$
1655	<0.1	$NPM1^{mut}/FLT3^{ITD}$	None	CD33+/CD13- , CD15-/HLA-DR-	$NPM1^{wt}/FLT3^{wt}$	$NPM1^{mut}/FLT3^{ITD}$
1719	<0.1	$FLT3^{ITD}$	None	CD133+/CD34-, CD15-/HLA-DR-	$FLT3^{wt}$	$FLT3^{ITD}$
1738	<0.1	$NPM1^{mut}$	None	CD33+/CD13-, CD15-/HLA-DR-	$NPM1^{wt}$	$NPM1^{mut}$
1894	<0.1	$NPM1^{mut}/FLT3^{ITD}$	None	CD33+/CD13-, CD15-/HLA-DR-, CD56	$NPM1^{wt}/FLT3^{wt}$	$NPM1^{mut}/FLT3^{ITD}$
2207	<0.1	$NPM1^{mut}$	None	CD133+/CD34- CD15-/HLA-DR-	$NPM1^{wt}$	$NPM1^{mut}$
CD34pos						
526	<0.1	$NPM1^{mut}/FLT3^{ITD\ (2x)}$	*CD33+	CD7, CD33+/CD13-, CD15-/HLA-DR-	$NPM1^{mut}/FLT3^{ITD\ (2x)}$	$NPM1^{mut}/FLT3^{ITD\ (2x)}$
575	0.1	$NPM1^{mut}$	*CLL-1	ND	$NPM1^{mut}$	$NPM1^{mut}$
945	55.0	$NPM1^{mut}$	CD7, CD19		$NPM1^{mut}$	$NPM1^{mut}$
951	37.0	$FLT3^{ITD}$	*CLL-1	ND	$FLT3^{ITD}$	$FLT3^{ITD}$
1263	10.0	$FLT3^{ITD}$	None	CD15-/HLA-DR-	$FLT3^{ITD}$	$FLT3^{ITD}$
1264	60.0	$FLT3^{ITD}$	CD13+/CD33-	CD13+/CD33-	$FLT3^{ITD}$	$FLT3^{ITD}$
1321	<0.1	$NPM1^{mut}$	*CD33+	CD15-/HLA-DR-, CD33+/CD13-	$NPM1^{mut}$	$NPM1^{mut}$
2005	5.7	$FLT3^{ITD\ (2x)}$	None	None	$FLT3^{ITD\ (2x)}$	ND
2315	<0.1	$NPM1^{mut}$	CD13+/CD33-	CD133+/CD34-	$NPM1^{mut}$	$NPM1^{mut}$

ND, not determined; mut, mutation; wt, wild type: no $FLT3^{ITD}$ or NPM1 mutation present. *Aberrant expression of CD33 or CLL-1 on CD34+CD38- blasts.

TABLE SIII | Univariate and Multivariate Cox Regression Analysis of Relapse-Free Survival and Overall Survival

Variable	No.	Univariate analysis			Multivariate analysis			No.	Univariate analysis			Multivariate analysis		
		Relapse incidence			Relapse incidence				Death incidence			Death incidence		
		HR	95% CI	p	HR	95% CI	p		HR	95% CI	p	HR	95% CI	p
Total	455							566						
CD34 status				<.001			.01				<.001			.02
CD34-negative	114							128						
CD34-positive	341	2.29	1.54-3.40		1.81	1.15-2.85		438	2.22	1.60- 3.07		1.57	1.08-2.29	
Age				.25			.17				.06			.05
<40	119							141						
>40	336	1.22	0.87-1.72		1.30	0.90-1.87		425	1.30	0.99- 1.71		1.34	1.00-1.79	
AML type				.13			.32				.82			.88
De Novo	367							458						
Secondary	42	1.58	1.01-2.48		1.33	0.83-2.13		52	1.13	0.77-1.67		1.05	0.71-1.56	
MDS	46	1.03	0.63-1.67		1.21	0.72-2.05		56	1.01	0.69-1.48		0.92	0.61-1.37	
CR reached				<.001			<.001				<.001			<.001
Never CR	0							111						
Early CR#	353							353	0.13	0.10- 0.19		0.30	0.21-0.42	
Late CR	102	2.05	1.49-2.81		2.23	1.60-3.11		102	0.25	0.17- 0.37		0.49	0.33-0.74	
Risk group AML				<.001			<.001				<.001			<.001
Good	101							107						
Intermediate	297	1.89	1.25-2.85		1.58	1.00-2.49		355	3.18	2.06- 4.90		2.03	1.27-3.25	
Poor	57	4.27	2.58-7.07		3.99	2.30-6.92		104	8.12	5.10-12.91		4.24	2.55-7.03	

Variable	No.	Univariate analysis			Multivariate analysis			No.	Univariate analysis			Multivariate analysis		
		Relapse incidence			Relapse incidence				Death incidence			Death incidence		
		HR	95% CI	p	HR	95% CI	p		HR	95% CI	p	HR	95% CI	p
FLT3/NPM1 status				<.001			.02				<.001			.13
FLT3^{wt} / NPM1^{wt}	271							351						
FLT3^{wt} / NPM1^{mut}	89	0.38	0.24-0.61		0.51	0.30-0.88		96	0.33	0.21- 0.50		0.73	0.44-1.21	
FLT3^{ITD} / NPM1^{wt}	41	1.51	0.96-2.38		1.47	0.91-2.35		55	1.33	0.94- 1.88		1.26	0.87-1.83	
FLT3^{ITD} / NPM1^{mut}	54	1.04	0.67-1.63		1.14	0.69-1.90		64	1.24	0.88- 1.72		1.35	0.92-1.99	
WBC at diagnosis (x 10^9/L)				.003			.01				<.001			.01
<20	271							325						
20-100	140	0.91	0.65-1.27		1.22	0.85-1.75		177	1.00	0.78- 1.30		1.08	0.82-1.43	
>100	44	1.98	1.29-3.05		2.06	1.28-3.32		64	1.89	1.37- 2.62		1.76	1.22-2.52	
Last consolidation therapy				<.001			<.001				<.001			<.001
None	106							217						
Cycle 3	93	0.50	0.34-0.75		0.62	0.41-0.94		93	0.24	0.15- 0.37		0.45	0.28-0.73	
Autologous SCT	80	0.34	0.22-0.53		0.37	0.23-0.59		80	0.22	0.14- 0.36		0.39	0.24-0.64	
Allogeneic SCT	176	0.22	0.15-0.34		0.16	0.10-0.25		176	0.38	0.28- 0.52		0.53	0.37-0.75	

In the multivariate analysis all known clinical risk factors were included. Individual mutations with clinical importance (Core binding factor AML, mutated CEBPα and EVI1) are incorporated in the risk groups. Last consolidation treatment was included as time-dependent covariate for both the RFS and OS analyses. CR status was only included as time-dependent variable in the OS analysis, since RFS was measured from date of CR. Abbreviations: CI, confidence interval; CR, complete remission; HR, hazard ratio, na, not applicable; SCT, stem cell transplantation; WBC, white blood cell count.

[#] Defined as CR after the 1st cycle of chemotherapy.

TABLE SIV | Additional value of CD34 in different subsets of AML patients based on risk and FLT3/NPM1 mutational profile

Group	N (Number)	% of total	3yr EFS % (SE)	3yr OS % (SE)
I Good risk (n=118)				
CD34neg + NPM1^{mut}/FLT3^{wt}	29	25	84 (7)	89 (6)
CD34pos + NPM1^{mut}/FLT3^{wt}	18	15	59 (12)	67 (13)
CD34neg + NPM1^{wt}/FLT3^{itd}	0	0	np	np
CD34pos + NPM1^{wt}/FLT3^{itd}	4	3	25 (22)	50 (25)
CD34neg + NPM1^{mut}/FLT3^{itd}	0	0	np	np
CD34pos + NPM1^{mut}/FLT3^{itd}	0	0	np	np
CD34neg + NPM1^{wt}/FLT3^{wt}	0	0	np	np
CD34pos + NPM1^{wt}/FLT3^{wt}	58	49	51 (7)	74 (6)
Missing	9	8		
II Intermediate risk (n=456)				
CD34neg + NPM1^{mut}/FLT3^{wt}	36	8	57 (8)	67 (8)
CD34pos + NPM1^{mut}/FLT3^{wt}	15	3	67 (12)	66 (12)
CD34neg + NPM1^{wt}/FLT3^{itd}	5	1	60 (22)	60 (22)
CD34pos + NPM1^{wt}/FLT3^{itd}	39	9	17 (6)	24 (7)
CD34neg + NPM1^{mut}/FLT3^{itd}	23	5	55 (11)	61 (11)
CD34pos + NPM1^{mut}/FLT3^{itd}	41	9	20 (6)	20 (6)
CD34neg + NPM1^{wt}/FLT3^{wt}	29	6	45 (9)	62 (9)
CD34pos + NPM1^{wt}/FLT3^{wt}	180	40	33 (4)	46 (4)
Missing	88	19		
III Poor risk (n=132)				
CD34neg + NPM1^{mut}/FLT3^{wt}	0	0	np	np
CD34pos + NPM1^{mut}/FLT3^{wt}	0	0	np	np
CD34neg + NPM1^{wt}/FLT3^{itd}	1	1	nr	nr
CD34pos + NPM1^{wt}/FLT3^{itd}	8	6	13 (12)	25 (15)
CD34neg + NPM1^{mut}/FLT3^{itd}	0	0	np	np
CD34pos + NPM1^{mut}/FLT3^{itd}	1	1	nr	nr
CD34neg + NPM1^{wt}/FLT3^{wt}	9	7	11 (11)	17 (14)
CD34pos + NPM1^{wt}/FLT3^{wt}	90	68	9 (3)	15 (4)
Missing	23	17		

EFS, event free survival; Np, not present; Nr, not reached; OS, overall survival; SE, standard error.

Supplementary figures

Distribution of CD34 percentage based on CD34 status in the total group of patients (A) and in patients with a LAP present (B)

A. Total patient cohort	>1% CD34+ blasts	≤1% CD34+ blasts	Total
CD34-positive AML	504	44	**548** (77.6%)
CD34-negative AML	1	157	**158** (22.4%)
Total	**505** (71.5%)	**201** (28.5%)	**706** (100.0%)

B. Patient cohort with a LAP present	>1% CD34+ blasts	≤1% CD34+ blasts	Total
CD34-positive AML	475	42	**517** (80.2%)
CD34-negative AML	1	127	**128** (19.8%)
Total	**476** (73.8%)	**169** (26.2%)	**645** (100.0%)

C. Decision tree to determine CD34 status

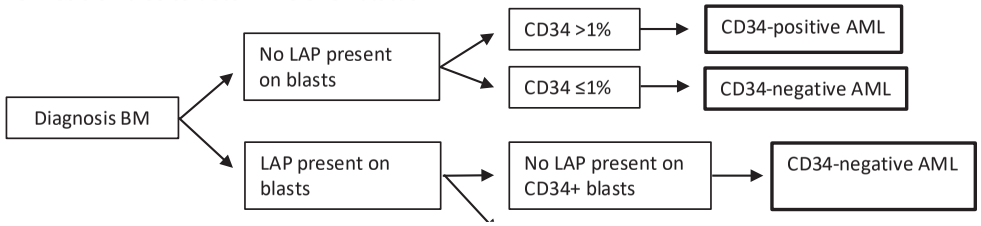

FIGURE S1 | Determination of CD34 status in AML patients.
Distribution of CD34 percentage between CD34-negative patients and CD34-positive patients in the total patient cohort (A) and in all patients with an aberrant immunophenotype present on the blasts (B). Importantly, a small group of CD34-positive patients (44/548; 8.0%) had CD34 ≤1% yet harbored neoplastic cells within the CD34+ blast compartment (A). Note that almost all CD34-negative patients in which a LAP was present (127/128) had a CD34 percentage ≤1% (B). (C) Decision tree to determine CD34 status based on both CD34 expression percentage and presence of LAPs.

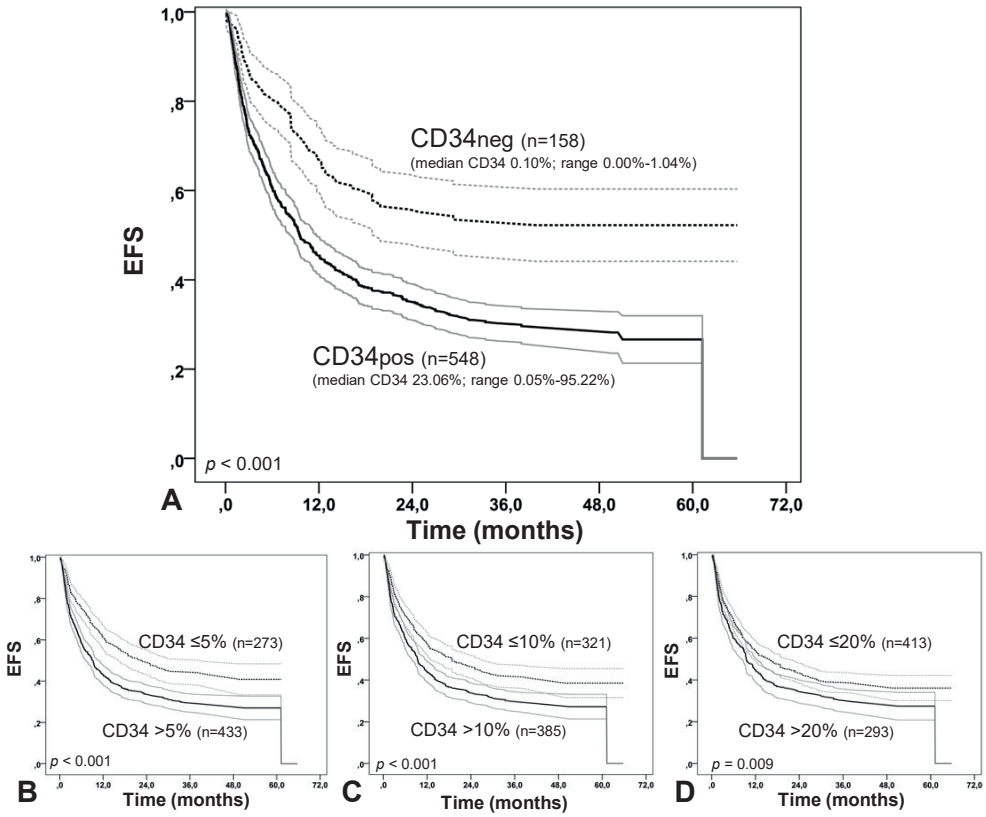

FIGURE S2 | Prognostic value of CD34 at diagnosis on EFS

This figure shows four Kaplan-Meier curves and associated 95% confidence intervals (in light gray) for EFS: A) our new approach to define CD34-positive and negative patients without structural use of a cut-off value; B) using a 5% cut-off value; C) using a 10% cut-off value and D) using a 20% cut-off value (D), all to define CD34 low and CD34 high patients. The new definition of CD34 shows the strongest prognostic impact. Fig S3 shows the same results for OS.

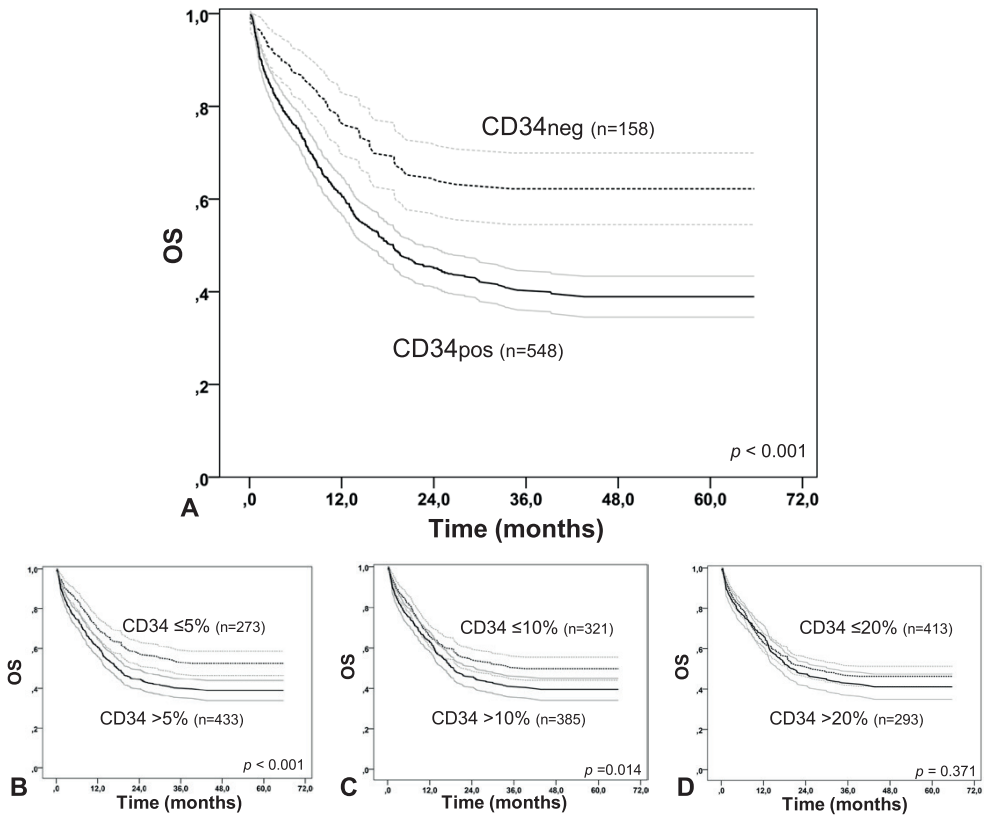

FIGURE S3 | Prognostic value of CD34 at diagnosis on OS

This figure shows four Kaplan-Meier curves (black lines) and associated 95% confidence intervals (light gray lines) for OS whereby different approaches are used to estimate the prognostic relevance of CD34: A) our new approach to define CD34-positive and negative patients without structural use of a cut-off value, B) using a 5% cut-off value, C) using a 10% cut-off value and D) using a 20% cut-off value to define CD34 low and CD34 high patients.

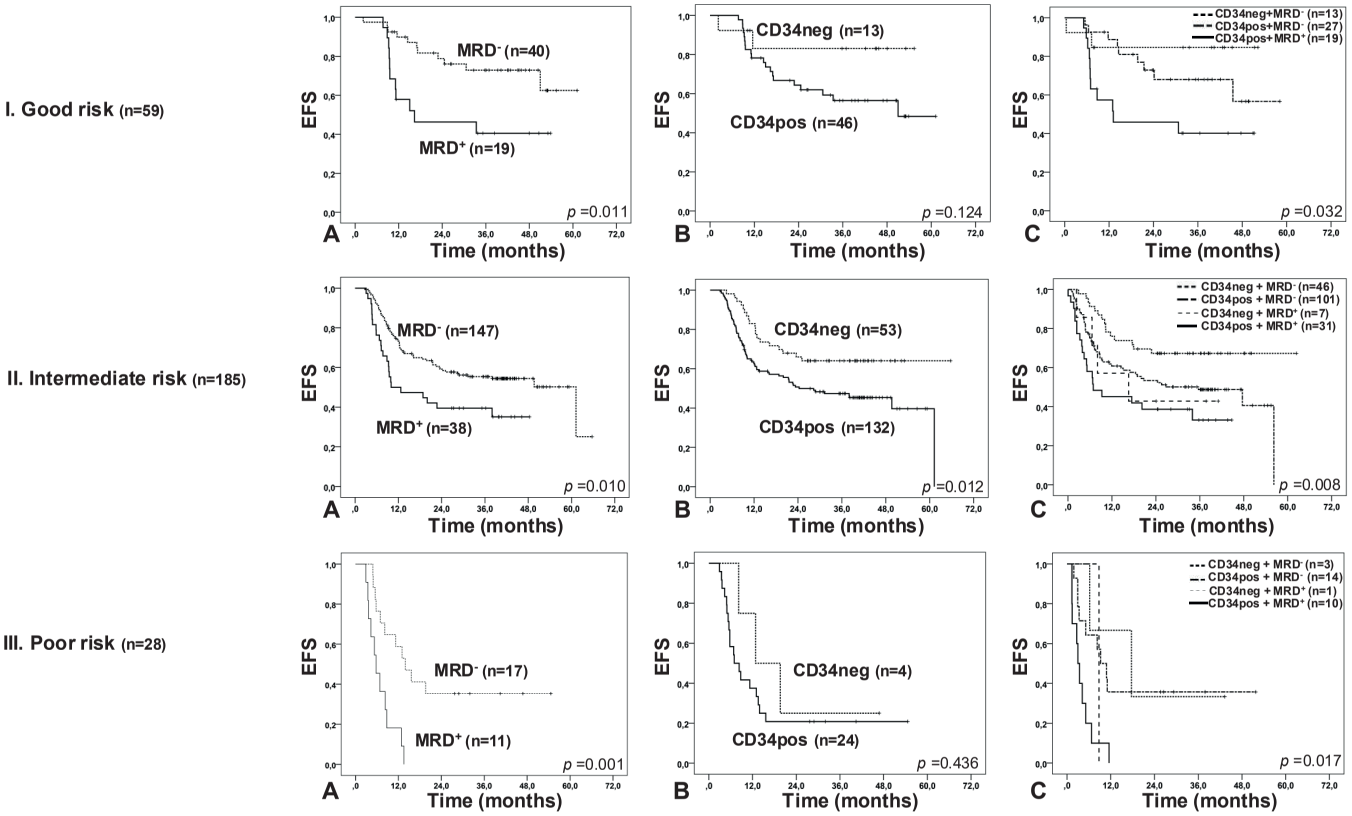

FIGURE S4. Prognostic value of CD34 combined with MRD in different risk categories.
Kaplan-Meier analyses for EFS in 59 good risk (I), 185 intermediate risk (II) and 28 poor risk (III) patients for which also MRD results after induction therapy (A) and CD34 status at diagnosis (B) were present. Note that the patient groups in which both CD34 and MRD were determined, has fewer patients compared to the total CD34 subgroups of Fig 5 since the MRD assessment after cycle II had not been done in all patients. Combining MRD and CD34 status resulted in 4 patient groups (C): 1) CD34neg/MRD$^-$, 2) CD34neg/MRD$^+$, 3) CD34pos/MRD$^-$ and 4) CD34pos/MRD$^+$. 1) Note that MRD further divides the CD34-positive group within the good and intermediate risk patients (I.A-C, II.A-C); 2) In the poor risk group MRD splits up the CD34-positive group, but OS remains low (III.C).